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Nuclear Receptor Expression Links the Circadian Clock to Metabolism

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SUMMARY

As sensors for fat-soluble hormones and dietary lipids, oscillations in nuclear receptor (NR) expression in key metabolic tissues may contribute to circadian entrainment of nutrient and energy metabolism. Surveying the diurnal expression profiles of all 49 mouse nuclear receptors in white and brown adipose tissue, liver, and skeletal muscle revealed that of the 45 NRs expressed, 25 are in a rhythmic cycle and 3 exhibit a single transient pulse of expression 4 hr into the light cycle. While thyroid hormones are generally constant, we find that *TR*α and β dramatically cycle, suggesting that fundamental concepts such as "basal metabolism" may require reexamination. The dynamic but coordinated changes in nuclear receptor expression, along with their key target genes, offers a logical explanation for known cyclic behavior of lipid and glucose metabolism and suggests novel roles for endocrine and orphan receptors in coupling the peripheral circadian clock to divergent metabolic outputs.

INTRODUCTION

The physiology and behavior of organisms across the animal kingdom adapt to 24 hr light/dark (LD) cycles, which in turn are controlled by evolutionarily conserved intrinsic circadian oscillators. In mammals, the central circadian clock, which resides in the suprachiasmatic nucleus (SCN) of the hypothalamus, is entrained directly by the daily LD cycle. This master clock is proposed to synchronize slave oscillators in peripheral tissues by yet-to-be-identified neuronal and hormonal mechanisms (Reppert and Weaver, 2002; Schibler and Sassone-Corsi, 2002), allowing animals to adapt their feeding, activity, and metabolism to predictable daily changes in the environment.

The central and peripheral oscillators share a common molecular circuitry. The clockwork comprises a battery of transcriptional activators and repressors that are "wired" into an autoregulatory transcriptional feedback loop. Clock and Bmal1 are paired transcriptional activators that drive the expression of *Period* genes (*Per1, Per2,* and *Per3*), *Cryptochrome* genes (*Cry1* and *Cry2*), and the orphan nuclear receptor *Rev-erb*α gene. Subsequently, the Per-Cry protein complex inhibits the transcription of its own genes, while Rev-erbα represses the expression of *Bmal*. This cell-autonomous feedback loop permits cyclic expression of these oscillator genes at various phases with the same period length of approximately 24 hr (Dunlap, 1999; King and Takahashi, 2000; Reppert and Weaver, 2002). Precisely how the circadian clock acts to control metabolic rhythms is not known, although the daily cycling of steroid hormones such as cortisol suggests a key role for nuclear receptors in this process.

The nuclear receptor (NR) superfamily is comprised of 49 members in mouse, which, in addition to regulating development and reproduction, coordinate diverse aspects of organ physiology. By sensing fat-soluble hormones, vitamins, and dietary lipids, NRs direct a wide range of molecular genetic programs that regulate lipid and carbohydrate metabolism (Chawla et al., 2001). It is well known that general metabolism is subject to rhythmic fluctuation in accordance with the LD cycle and logically speculated that circadian variations in the levels of NR hormones may trigger metabolic rhythms. However, a critical question yet to be addressed is whether the expression levels of NRs per se fluctuate in a circadian manner so as to elicit metabolic rhythms.

The Nuclear Receptor Signaling Atlas (NURSA) is a consortium of scientists investigating NR action using a systems-wide approach (www.nursa.org). As part of this effort we began by quantifying the anatomical expression of all 49 NRs in 39 different mouse tissues (see Bookout et al., 2006 [this issue of *Cell*]). Here we present a detailed analysis of the circadian expression of NRs in liver, skeletal muscle, white adipose tissue (WAT), and brown adipose tissue (BAT), all of which represent the major peripheral

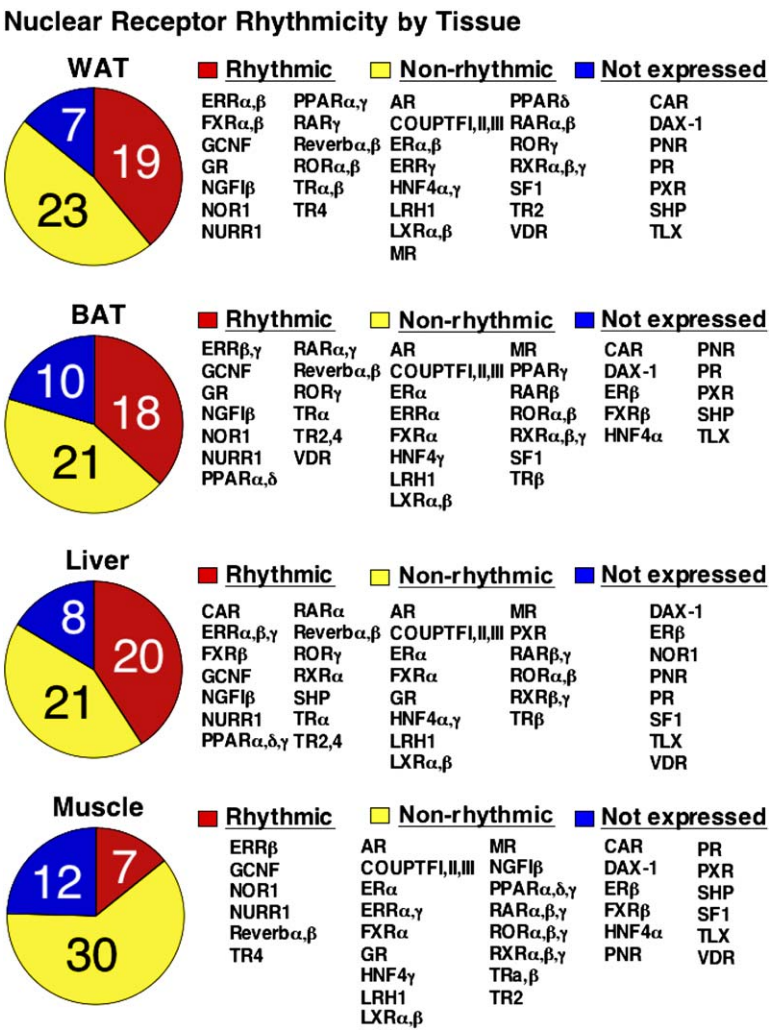

Figure 1. Rhythmicity of Nuclear Receptor Expression Profiles in Four Metabolic Tissues

Pie-graph analysis of the distribution of nuclear receptor expression in white adipose tissue (WAT), brown adipose tissue (BAT), liver, and skeletal muscle. The 49 mouse nuclear receptors are categorized according to their expression and rhythmicity in each tissue.

sites that integrate energy flux to meet the physiological needs of the body. Our results reveal broad expression (45 NRs) with 28 NRs displaying tissue-specific oscillation in these metabolically active tissues. Nuclear receptor rhythmicity offers possible explanations for diurnal fluctuations in lipid and glucose metabolism and may represent an unexpectedly large scale coordination of signaling pathways that contribute to this process. This study suggests that the nuclear receptor superfamily comprises a wealth of clock-controlled genes that relay temporal and nutritional cues to control metabolic physiology. The complete data sets are available on the NURSA website (www.nursa.org/datasets.cfm?doi=10.1621/datasets.02002).

RESULTS AND DISCUSSION

Extensive Expression of the NR Superfamily in Metabolic Tissues

To study the temporal relationship between nuclear receptor expression and metabolic processes, we compiled a dynamic atlas of the NR expression in four key metabolic tissues (liver, muscle, brown adipose, and white adipose) over an LD cycle in C57BL/6J mice. The analysis of each receptor gene by two approaches—TaqMan and SYBR Green real-time PCR—yielded largely consistent results. The complete colorized data sets are available at the NURSA website (www.nursa.org) and on the accompanying poster.

Our results identified a surprisingly large number of NRs (45) that are expressed in at least one of the four key metabolic tissues, with a range of 37 in muscle to 42 in WAT (Figure 1). Within this remarkable abundance of signaling pathways, a few NRs showed expression restricted to a particular tissue, like the xenobiotic receptors pregnane X receptor (*PXR*) and constitutive androstane receptor (*CAR*) in liver and the estrogen receptor β (*ERβ*) in WAT (Figure 1). This diversity of NR expression in each tissue suggests that the nuclear receptor superfamily may act as a megagenetic entity to influence metabolism rather than a series of independent signaling pathways. Additional evidence for this point emerges from an analysis of the cycling patterns of each receptor.

To address the potential involvement of NRs in peripheral circadian circuits, we analyzed their temporal rhythmicity using a cosine wave-fitting algorithm (COSOPT) (Panda et al., 2002; Straume, 2004). This analysis revealed that 28 NR genes (representing 57% of the superfamily) exhibit circadian-like patterns of expression. WAT, BAT, and liver express a greater number of periodically expressed NR genes (19, 18, and 20, respectively), whereas the number of cyclic genes in skeletal muscle is significantly less (7) and includes only nonligand-dependent orphan receptors (Figure 1). The cycling genes can be subgrouped based on tissue selectivity with six receptors (estrogen-related receptor β [*ERRβ*], germ cell nuclear factor (*GCNF*), *NURR1*, *Rev-erbα*, *Rev-erbβ*, and testicular receptor 4 [*TR4*]) rhythmically expressed in all four tissues. Fourteen others (*ERRα*, *ERRγ*, farnesoid X receptor β [*FXRβ*], glucocorticoid receptor [*GR*], *NGFI-B*, *NOR1*, peroxisome proliferator-activated receptor α [*PPARα*], *PPARγ*, *PPARδ*, retinoic acid receptor α (*RARα*), *RARγ*, retinoic acid receptor-related orphan receptor γ (*RORγ*), *TR2*, and thyroid hormone receptor α (*TRα*) selectively cycle in two or three tissues, and seven receptors (*FXRα*, *RORα*, *RORβ*, *TRβ*, vitamin D receptor [*VDR*], *CAR*, and *SHP*) cycle in a single tissue (Figure 1). This initial overview indicates that a large proportion of NRs are subject to regulation by the circadian clock.

The further assessment of rhythmic NRs reveals a nonrandom distribution of their peak expression throughout the circadian cycle (Table 1). The largest numbers of NR transcripts peak at *Zeitgeber* Time (ZT) 4 in all four tissues ascribing a special regulatory importance to this time point. Among the NRs that cycle in at least two tissues, eight (*ERRα*, *ERRγ*, *NGFI-B*, *NOR1*, *Rev-erbα*, *Rev-erbβ*, *RORγ*, and *TRα*) display synchronized rhythms in different tissues, while others are decoupled from each other (Table 1). Decoupling is unusual, suggesting that the cycling of some NRs may be entrained by various *Zeitgebers*, creating distinct temporal organization of local physiologic responses.

Oscillation of Circadian Clock and NRs in Metabolic Tissues

To begin to understand the relationship between the various *Zeitgebers* and NR gene expression, we surveyed the expression of the major known oscillator genes (*Bmal1*, *Clock*, *Per2*, *Cry1*, and *Rev-erbα*) in the four metabolic tissues (Figure 2). While the existence of the circadian clock in liver and muscle has been previously described (Panda et al., 2002; Storch et al., 2002; Zambon et al., 2003), we extend the observation to show that the oscillator genes are each expressed in all four tissues displaying the same rhythmic patterns. Consistent with previous reports (Dunlap, 1999; Okamura et al., 1999; Preitner et al., 2002), the expression of *Bmal1*, *Per2*, *Cry1*, and *Rev-erbα* exhibited robust cycling, while only weak rhythmicity was observed for clock expression. Of note, *Per2* mRNA reached the zenith at dusk when *Bmal1* mRNA was at its nadir in all four tissues (Figure 2). Our results provide the first evidence for the presence of a molecular oscillator in adipose tissue (WAT and BAT) and reveal their entrainment with liver and muscle. Intriguingly, the circadian-regulated NRs generally fall within specific NR subfamily groups. For example, all members of the *ROR* (α, β, γ), *Rev-erb* (α and β), *PPAR* (α, γ, δ), *FXR* (α and β), *TR* (α and β), *ERR* (α, β, γ), *NGFI-B* (*NGFI-B*, *NOR1*, *NURR1*), and *TR2/TR4* subfamilies show circadian expression.

Rev-erbβ and RORs: Potential Novel Oscillator Components

Rev-erb and ROR subfamilies comprise closely related orphan NRs that recognize similar *cis* response elements (*RORE*s) on target genes (Forman et al., 1994). RORs are known as constitutive transcriptional activators, whereas Rev-erbs function as constitutive repressors (Dumas et al., 1994; Forman et al., 1994; Retnakaran et al., 1994). It has been postulated that the crosstalk between these two families of NRs forms a regulatory network. Indeed, several genes, such as α-*fetoprotein* and *N-myc*, have been described as targets of both RORα and Rev-erbs (Bois-Joyeux et al., 2000; Dussault and Giguere, 1997).

Coexpression of *Rev-erb* and *ROR* genes within the same tissue is prerequisite for direct crosstalk. Consistent with previous studies (Panda et al., 2002; Preitner et al., 2002; Triqueneaux et al., 2004; Ueda et al., 2002), our results show that the expression of *Rev-erbα* and -β in the liver display overt rhythmicity with the peak level at ZT 4 and ZT 8, respectively. Importantly, as seen with the core clock genes, oscillating phases of *Rev-erbα* and -β were present across all four tissues (Figure 2). This result suggests that, like *Rev-erbα*, *Rev-erbβ* may also serve as a novel component of the molecular clock.

Several lines of evidence suggest that the ROR family is also closely associated with the circadian clock. Rev-erbα has been identified as a major repressor of Bmal1 transcription via two *ROR* response elements (*RORE*s) in its promoter (Preitner et al., 2002). Recently it was shown that that RORα can activate Bmal1 transcription via the same *RORE*s (Akashi and Takumi, 2005; Sato et al., 2004). Interestingly, the promoter of the *Rev-erbα* gene harbors a functional *RORE* through which it is repressed by itself and induced by RORα (Delerive et al., 2002; Raspe et al., 2002). Therefore, RORα is implicated in the regulation of two well-established clock genes. Here we show that, in contrast to the uniform circadian patterns of both *Rev-erbs* in all four tissues, the expression of *ROR* isoforms exhibit distinct tissue-specific patterns. *RORα* expression is rhythmic in WAT but not in BAT, liver, and muscle, whereas cyclic expression of *RORγ* is specific for BAT and liver (Figure 2). *RORβ* is poorly expressed in these four peripheral tissues (data not shown). *ROR* isoforms also differ in their temporal patterns of expression. *RORα* levels peaked at ZT 12 in WAT, while *RORγ* reached its maximum level at ZT 16 in BAT and liver. Taken together, we propose that the RORs may serve as accessory oscillator components that help transform

Table 1. Peak Expression of Rhythmic Nuclear Receptors in Metabolic Tissues

	ZT0	ZT4	ZT8	ZT12	ZT16	ZT20
WAT	GCNF	NGFI-B	PPARα	GR	ERRα	ERRβ
	RORβ	NOR1	Rev-erbβ	RARγ	FXRα	FXRβ
		NURR1		RORα	PPARγ	
		Rev-erbα				
		TR4				
		TRα				
		TRβ				
BAT	GR	GCNF	ERRγ		RORγ	ERRβ
	PPARδ	NGFI-B	Rev-erbβ			
	TR4	NOR1				
		NURR1				
		PPARα				
		RARα				
		RARγ				
		Rev-erbα				
		TR2				
		TRα				
		VDR				
Liver	FXRβ	ERRβ	ERRγ	CAR	ERRα	NURR1
		NGFI-B	GCNF	PPARα	RORγ	PPARδ
		Rev-erbα	PPARγ			
		TR2	RARα			
		TR4	Rev-erbβ			
		TRα	SHP			
Muscle		ERRβ	Rev-erbβ	GCNF		
		NOR1		TR4		
		NURR1				
		Rev-erbα				

timing information from the core clock into tissue-specific responses.

PPARs: Potential Link between the Circadian Clock and Energy Metabolism

The three PPAR family members have been shown to regulate lipid metabolism and energy homeostasis by coordinated actions in adipose tissue, liver, and muscle (Evans et al., 2004; Lee et al., 2003). PPARγ is most abundant in adipose tissue, where it activates transcriptional programs for lipid storage and lipogenesis (He et al., 2003). PPARα is known for its role in promoting hepatic fatty acid oxidation and ketogenesis in response to fasting (Kersten et al., 1999; Leone et al., 1999). PPARδ is more ubiquitously present (Figure 3A).

Despite our growing understanding of the complementary functions of PPARs in distinct metabolic sites, little is known as to whether the temporal fluctuation of metabolism over the daily LD cycle might be paralleled by changes in *PPAR* expression (Inoue et al., 2005; Lemberger et al., 1996; Oishi et al., 2005) Here we show that the expression of *PPARs* exhibit characteristic but distinct tissue-specific rhythms (Figure 3). For example, *PPARα* transcripts cycle in WAT, BAT, and liver but not in muscle. *PPARγ* selectively cycles in WAT and liver, whereas *PPARδ* transcripts oscillate only in BAT and liver (Figure 3). Furthermore, there are significant variations in PPAR oscillations between different tissues. For instance, the peaks for *PPARα* levels are staggered by approximately 4 hr between BAT, WAT, and liver (Figure 3A). Some of these circadian patterns correlate well with known functions of PPARs. In nocturnal rodents such as mice, *PPARα* is induced during the daytime when they are generally in a fasting state that requires ketone bodies and is largely

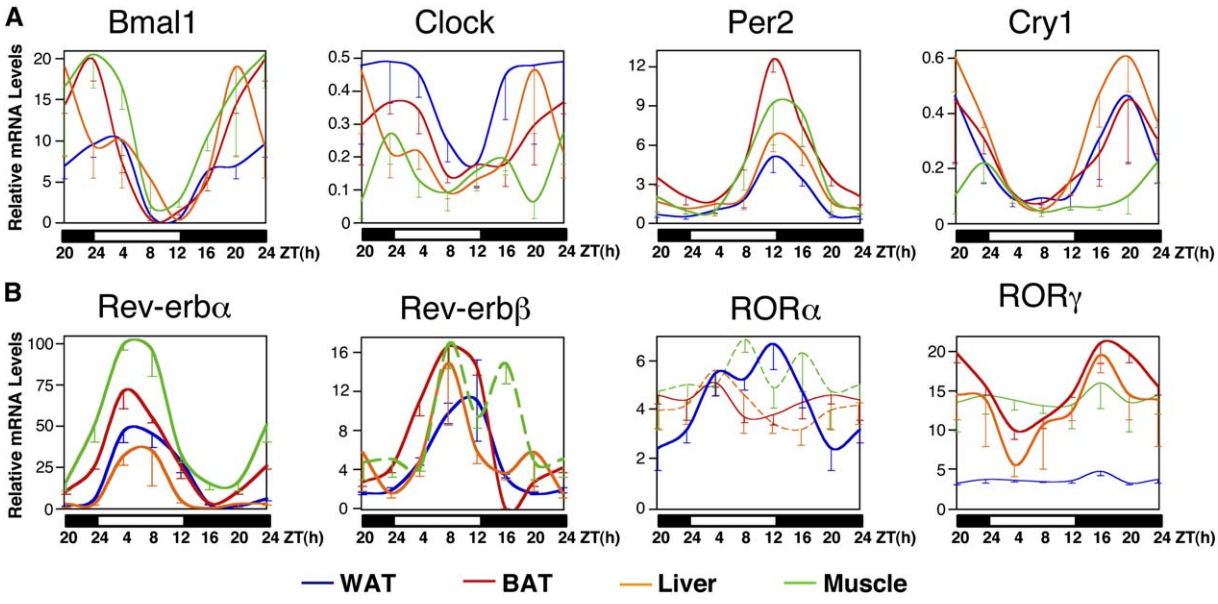

Figure 2. Expression of Core Clock Genes, Rev-erb, and ROR Families in Peripheral Tissues

Mice were entrained to a 12 hr light, 12 hr dark cycle. WAT, BAT, liver, and skeletal muscle tissues were collected from four mice per time point at *Zeitgeber* time (ZT) 0 (24), 4, 8, 12, 16, and 20. Lights were turned on and off at ZT 24 and ZT 12, respectively.

(A) Levels of *Bmal1*, *Clock*, *Per2*, and *Cry1* mRNA in the four tissues were quantified by TaqMan real-time RT-PCR. Values represent the mean of relative RNA levels from four individual mice and error bars depict SD.

(B) TaqMan real-time PCR analysis of *Rev-erbα*, *Rev-erbβ*, *RORα*, and *RORγ* mRNA levels in indicated tissues over a 24 hr LD cycle. The relative levels of Rev-erbβ muscle, and RORα muscle and liver expression (dashed lines) are 2.5-, 4-, and 2-fold the value of the scale shown, respectively, and error bars depict SD. Relative mRNA levels were expressed as ratios relative to internal 36B4 mRNA levels. Timepoints ZT 20 and 24 have been duplicated in all of the graphs to facilitate viewing of the time curve.

inhibited at night when feeding provides hexose sugars as a primary fuel source.

To identify which genes in adipose tissue might be under circadian control, we examined several key regulators, including PPARγ, sterol regulatory element binding protein-1 (SREBP-1), and CCAAT/enhancer binding protein (C/EBP). Both *PPARγ* and *SREBP-1c* are rhythmically expressed in WAT and peak concurrently at ZT 16 (Figure 3B); *C/EBPα* and *-β* do not cycle (Figure S1). A subset of adipose-specific genes, including *adiponectin* and *leptin*, fluctuate in phase with *PPARγ* and *SREBP-1c* transcripts (Figure 3B), which have been established as positive regulators of these genes (Seo et al., 2004), suggesting that oscillation of the regulators may directly determine the rhythmicity of certain target genes.

Adipose tissue is also involved in thermogenesis through uncoupling protein (UCP)-mediated energy dissipation (Tiraby and Langin, 2003). The observed cycling of *PPARδ* in BAT correlates with that of several thermogenic genes, including *UCP-1* (Figure 3C), suggesting that PPARδ may link diurnal variations in body temperature to the intrinsic circadian clock.

The action of nuclear receptors in energy metabolism is mediated by a number of coregulators, including PGC-1α and the p160 family (SRC-1, SRC-2, and SRC-3) (McKenna and O'Malley, 2002; Picard et al., 2002; Tiraby and Langin, 2003). Although all members of the p160 family are expressed in WAT, BAT, liver, and muscle, there is no evidence for rhythmicity (Figure S2). In contrast, *PGC-1α* shows robust expression in BAT and, more interestingly, is rhythmically expressed in phase with *PPARα*, *RARα*, *TRα*, and *UCP1* (Figure 3D). This reinforces the idea that a coordinated oscillation of NRs and coregulators drives the expression of certain key targets. This is especially relevant since the levels of PGC-1, by activating the NRs, is directly linked to the induction of downstream target genes. PGC-1α has been known to play a central role in cold and diet-induced thermogenesis (Lowell and Spiegelman, 2000). However, the accumulation of *PGC-1α* and *UCP1* transcripts in the morning (ZT 0–4) is unlikely to be induced by either cold or diet, since the mice are maintained in a thermally stable environment and fed ad libitum. These results suggest a pathway as to how diurnal variations in body temperature may be linked to the intrinsic circadian clock. In aggregate, these observations clearly reveal that the circadian clock operates directly at the level of key regulatory genes to translate temporal cycles into body physiology.

ERRs: Novel Circadian Regulators in Metabolism

The ERR family includes three orphan receptor members that act as constitutive transcriptional activators (Hong et al., 1999; Xie et al., 1999). In the adult, both *ERRα* and *ERRγ* are widely expressed, whereas *ERRβ* expression

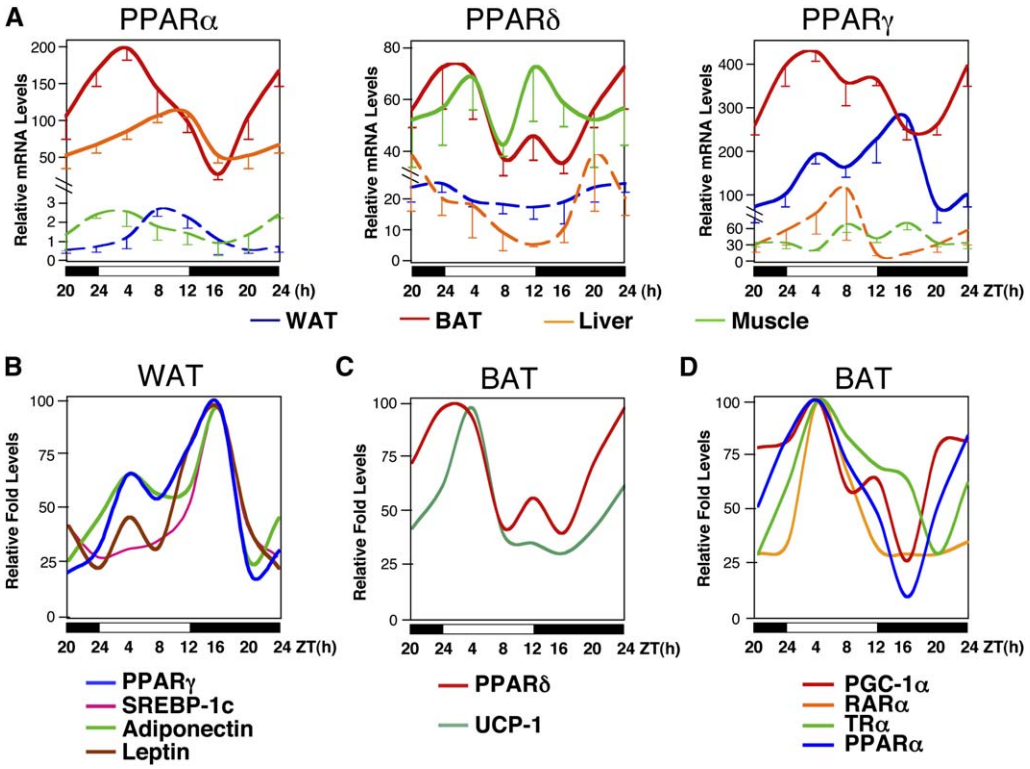

Figure 3. Tissue-Specific Diurnal Rhythm of PPARs and Target Gene Expression

(A) TaqMan real-time PCR analysis of *PPARα*, -γ, and -δ expression in indicated tissues over a 24 hr LD cycle. Relative mRNA levels were normalized against *36B4* mRNA levels. Values are represented as mean ± SD (n = 4).

(B) Rhythmic expression of *SREBP-1c* and *PPARγ* in WAT with peak at ZT 16. A similar pattern is observed for adiponectin and leptin in WAT.

(C) Correlating patterns of *PPARα* and *PPARδ* expression with that of *UCP1* in BAT.

(D) Rhythmic expression of *PGC-1α*, *RARα*, *TRα*, and *PPARα* in BAT resembling that of *UCP1* and *PPARδ*.

For (B)–(D), relative mRNA levels were normalized against *36B4* mRNA levels and subsequently normalized against a maximal value (set at 100) within the measured time points. Time points ZT 20 and 24 have been duplicated in all of the graphs to facilitate viewing of the time curve.

is restricted to tissues such as the eye, brain, thyroid, kidney, and heart (Bookout et al., 2006; Poster S1). Although ERRs may crosstalk with estrogen receptors (ERs) (Giguere, 2002; Horard et al., 2004; Horard and Vanacker, 2003), recent evidence indicates that ERRα may play a central role in regulating fatty acid metabolism and energy balance (Kamei et al., 2003; Luo et al., 2003; Mootha et al., 2004). In contrast, the roles of ERRβ and ERRγ in metabolism remain largely unknown.

Using quantitative PCR, we show that the transcripts of all *ERR* subtypes are detectable in the four peripheral tissues (Figure 4 and Poster S1). The expression of these subtypes exhibits distinct diurnal rhythmicity. The *ERRα* transcript selectively oscillates in WAT and liver, while *ERRγ* is cyclic in BAT and liver (Figure 4). Unexpectedly, *ERRβ* is rhythmically expressed in all four tissues, with higher levels at the subjective dawn and lower levels at the subjective dusk (Figure 4). This pattern suggests a potential role for ERRβ in coordinating oxidative metabolism in these tissues. The circadian expression of ERRs in these metabolically active tissues hints at their distinct or overlapping functions in energy metabolism. In support of this, the analysis of nuclear receptor expression in a broader range of tissues (Bookout et al., 2006) reveal that the three ERR isoforms are enriched in highly oxidative tissues such as those of the brain, heart, and kidney. In aggregate, these data suggest that all of the ERR family members may serve as direct molecular links between the circadian oscillator and energy metabolism.

The NGFI-B Family: Metabolic Implication of "STP" Rhythm

NGFI-B, NOR1, and NURR1 are three family members that recognize a common DNA element and activate gene expression in a ligand-independent manner. In cultured cells they mediate immediate-early responses to a broad variety of signals, and in vivo they are implicated in embryonic gastrulation, dopamine neurogenesis, and T lymphocyte apoptosis (Calnan et al., 1995; DeYoung et al., 2003; Lee et al., 1995; Zetterstrom et al., 1997). Nevertheless, the roles of these orphan receptors in the peripheral tissues, and particularly in metabolic function, have not been addressed. In the present study we found that all three are expressed at relatively high levels in

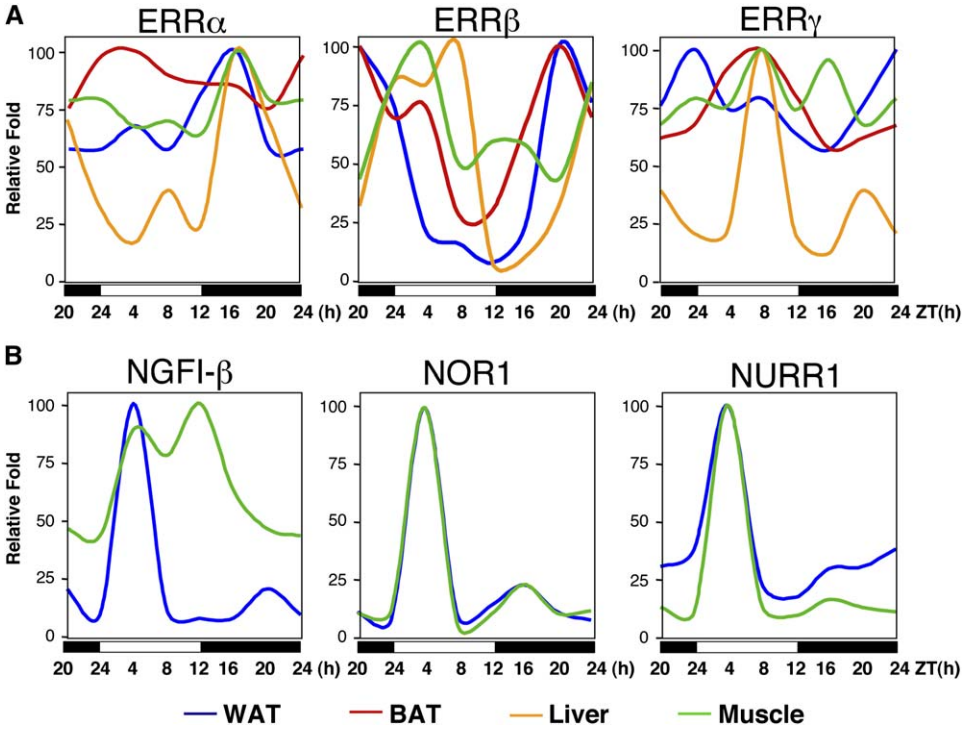

Figure 4. Diurnal Expression of ERR and NGFI-B Families

(A) Diurnal rhythm of *ERRα*, *-β*, and *-γ*.

(B) Representative data showing STP rhythm of *NGFI-B*, *NOR1*, and *NURR1*.

For all graphs, relative mRNA levels were normalized against *36B4* mRNA levels and subsequently normalized against a maximal value (set at 100) within the measured time points. Time points ZT 20 and 24 have been duplicated to facilitate viewing of the time curve.

BAT and muscle, at low levels in WAT, and at extremely low or undetectable levels in liver (Figure 4B). Analyses of their expression over an LD cycle revealed a unique rhythmic pattern in which their transcripts spike at ZT 4, followed by a precipitous decline in the next 4 hr, and remain at low levels through the rest of the day (Figure 4B). We refer to this unusual rhythm as a single transient pulse (STP). Since ZT 0 is the start of the light cycle, we speculate that light might serve as an indirect environmental cue that triggers the expression of the *NGFI-B* family genes in the peripheral tissues. Whatever the trigger is, these results reveal a previously unrecognized short-term synchronous pulse that has the potential of a previously unrecognized component of metabolic entrainment.

Perspectives

The principal role of the biological clock is to entrain a transcriptional network to synchronize the physiology and behavior of animals, such as sleeping, eating, reproduction, and metabolism, in alignment with daily and seasonal cycles of the earth. While some steroid receptors and their hormones have been linked to both circadian and reproductive rhythms, the present study was designed to bring a comprehensive, unbiased, and quantitative approach to NR expression in key metabolic tissues over a 24 hr LD cycle. By its nature the quantitative profiling focuses on the analysis of diurnal rhythmicity of the NR superfamily at the mRNA level. To gain better insight into circadian regulation by NRs, the rhythmicity of NRs at the protein level and their subcellular localization and activity will need to be explored. Finally, there is evidence that circadian-rhythm disorders might be associated with diabetes, obesity, and cardiovascular disease (Boethel, 2002; Turek et al., 2005). Our data suggest that nuclear receptors may serve to explain some aspects of the uncoupled rhythmicity linked to metabolic diseases. As nuclear receptors are targets of widely prescribed drugs, understanding their circadian patterns may shed some light on the development of chronotherapy and lead to enhanced drug efficacy through delivery timed to match peak receptor levels.

EXPERIMENTAL PROCEDURES

Animals

Male C57BL/6J mice of 7–8 weeks of age were purchased from Jackson Laboratory. Mice were maintained at a controlled temperature (23°C) on a 12 hr light/dark cycle (lights on 0600–1800) and provided water and standard rodent chow (Harlan Teklad) ad libitum. All animal care and use procedures were in accordance with institutional Animal Care and Use Committee guidelines. After being housed for 2 weeks, 24 mice were sacrificed by cervical dislocation at 4 hr intervals over 24 hr with four mice at each time point. Epididymal white adipose

tissue, interscapular brown adipose tissue, liver, and quadriceps muscle were dissected, rapidly frozen in liquid nitrogen, and stored at −80°C until RNA extraction. Total RNA for real-time PCR and Northern blotting was extracted using TRIZOL reagent (Invitrogen) according to the manufacturer's protocol.

High-Throughput Real-Time RT-PCR

Relative RNA levels were quantified by real-time RT-PCR technology with TaqMan and SYBR Green Reagents separately (Applied Biosystems). cDNA was synthesized from 2 μg of DNase-treated total RNA using Superscript II reverse transcriptase (Invitrogen). Primers and probes were designed using ABI PrimerExpress software. Sequences of primers and probes used in this study are shown in Tables S1 and S2. All probes for TaqMan real-time PCR were 5′ labeled with FAM (6-carboxyfluorescein) and 3′ labeled with TARMA (6-carboxytetramethylrhodamine). PCR reactions were assembled using a MultiPROBE II Robotic Liquid Handling System (Packard) containing 1× TaqMan Universal PCR Master Mix, 300 nM primers, 250 nM probe, and cDNA equivalent to 1 or 5 ng total RNA in a 10 μl volume. PCR was performed in an ABI PRISM 7700 detection system (Perkin Elmer) at 50°C for 2 min and 95°C for 10 min, followed by 40 two-step cycles of 95°C for 15 s and 60°C for 1 min. Relative mRNA levels were calculated using the comparative delta-Ct method and normalized against *36B4* mRNA levels in the same total RNA samples.

SYBR green fluorescence-based real-time PCR was performed in a procedure analogous to TaqMan technology. The primer sequences are identical to those used for TaqMan real-time PCR. PCR reactions contained 1× SYBR Green Master Mix (Applied Biosystems), 100 nM primers, and indicated amounts of cDNA. Thermal cycling parameters are in accordance with manufacturer's instructions (Perkin Elmer).

Statistical Analysis for Transcript Rhythmicity

A statistical program, COSOPT, was employed to define circadianly expressed transcripts (Panda et al., 2002; Straume, 2004). COSOPT imports data and calculates the mean expression intensity and its corresponding standard deviation (SD). Variable-weighting of individual time points are accommodated during analysis for the presence of rhythms at periods from 8 to 40 hr in 0.01 hr increments. For each test period, 101 test cosine basis functions (of unit amplitude) are considered, varying over a range of phase values from plus one-half the period to minus one-half the period. COSOPT calculates, for each test cosine basis function, the least-squares optimized linear correspondence between the experimental data, y(t), and the test cosine basis function, yb(t), as a function of time, t (i.e., such that the approximation of y(t) by the test cosine basis function, yb(t), is optimized across all values, t, in terms of two parameters, ALPHA and BETA, whereby $y(t) \sim APHA + BETA \times yb(t)$). The quality of optimization possible by the test cosine basis function is quantitatively characterized by the sum of squared residuals between y(t) and the approximation given by (ALPHA + BETA × yb(t)) (referred to as CHI2, for Chi-squared). The values of CHI2 are used to identify the phase at which the optimal correspondence between y(t) and yb(t) is obtained for each test period (i.e., the phase giving the smallest CHI2 value corresponds to the optimal phase). Thus, for each test period are assessed these values of ALPHA, BETA, and CHI2 at the optimal phase. One thousand Monte Carlo cycles are carried out, in which surrogate realizations of y(t) are generated by both (1) randomly shuffling temporal sequence and (2) adding pseudo-Gaussian-distributed noise to each surrogate point in proportion to the corresponding value of point-wise uncertainty (i.e., replicate SEM). Then, as with the original y(t) sequence, optimal values of ALPHA and BETA are determined, along with a corresponding CHI2, and retained in memory for each surrogate at each test period/optimal phase. The mean and SDs of the surrogate BETA values are then calculated, followed by the calculation of a one-sided significance probability based on a normality assumption. Multiple-measures correction of BETA values (pMMC-β) describes the goodness of fit. A significance threshold for pMMC-β was empirically estimated to accommodate known circadian clock genes.

Complete data sets and graphs for circadian expression of all 49 mouse nuclear receptors are available on the NURSA website (www.nursa.org/datasets.cfm?doi=101621/datasets.02002).

Supplemental Data

Supplemental Data include two figures, two tables, and one poster and can be found with this article online at http://www.cell.com/cgi/content/full/126/4/801/DC1/.

The supplemental poster shows a graphical representation of the anatomical and circadian expression profiles of the 49 mouse NRs from this work and the companion paper by Bookout et al. (2006). The poster depicts the common and IUPHAR nomenclature for each receptor, along with the chemical structure of known regulatory ligands. The center panel illustrates the phylogenetic tree of the NR superfamily.

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